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Bradykinin stimulates bone resorption and lysosomal-enzyme release in cultured mouse calvaria

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The effect of bradykinin on bone resorption was studied in cultures of newborn-mouse calvaria. Bradykinin (0.03 μM, 1 μM) stimulated the release of $^{45}Ca^{2+}$ from bones dissected out from mice prelabelled *in vivo* with ^{45}Ca. Bradykinin (1 μM) also augmented the release of stable calcium (^{40}Ca), P_i and the lysosomal enzyme β-glucuronidase. The stimulatory effect of bradykinin on mineral mobilization and lysosomal-enzyme release could be blocked by indomethacin. It is speculated that concomitant generation of thrombin and bradykinin in areas of trauma and inflammation may induce resorption of nearby bone tissue.

Several products synthesized and secreted by inflammatory cells can stimulate bone resorption in cultured foetal-rat long bones and newborn-mouse calvaria. Such agents may be implicated in the pathogenesis of bone resorption seen in rarifying forms of osteitis and osteomyelitis and in advanced lesions of chronic inflammatory processes such as periodontal disease and rheumatoid arthritis. Potential mediators of inflammatory induced bone resorption include osteoclast activating factor (OAF), synthesized by lymphocytes, prostaglandin E_2 (PGE_2) and interleukin-1 produced by monocytes/macrophages (Klein & Raisz, 1970; Horton *et al.*, 1972; Mundy, 1981; Gowen *et al.*, 1983). On the basis of our recent finding that thrombin can stimulate bone resorption in cultured mouse calvaria, we have proposed that products formed in the coagulation cascade may be involved in inflammation-induced bone resorption (Gustafson & Lerner, 1983). That study, as well as the present one, was stimulated by the current great interest in the role of coagulation factors in inflammation and immune injury. Thus it has been shown that macrophages can produce vitamin K-dependent coagulation factors and also thromboplastin (Prydz *et al.*, 1979; Østerud *et al.*, 1980; Lindahl *et al.*, 1982; Hogg, 1983). These findings have raised the possibility that a local generation of thrombin may be responsible for the deposition of fibrin seen in different types of inflammatory lesions (Riddle *et al.*, 1965; Colvin *et al.*, 1973; Andersen, 1980; Hopper *et al.*, 1981; Geczy & Meyer, 1982). Besides having many functions in the primary haemostasis and blood coagulation, thrombin also has direct cellular effects such as stimulation of mitotic activity in fibroblasts and endothelial cells (Chen & Buchanan, 1975; Baker *et al.*, 1979; Shuman *et al.*, 1981). In addition, thrombin has been shown to trigger the synthesis of prostaglandins in several cell types via interaction with specific cell-surface receptors (Hong & Levine, 1976; Hong *et al.*, 1976; Weksler *et al.*, 1978; Czervikone *et al.*, 1979; Hong, 1980).

Some of the cells that can be stimulated by thrombin also respond to bradykinin with a burst of prostaglandin synthesis (Hong & Levine, 1976; Hong *et al.*, 1976; Hong, 1980; Becherer *et al.*, 1982; Whorton *et al.*, 1982). Bradykinin, which is a small peptide, is formed by cleavage of kininogen by kallikrein (for a review, see Regoli & Barabé, 1980). Since pre-kallikrein is activated to kallikrein by activated Hageman factor (factor XIIa) and factor XII to factor XIIa by kallikrein (Meier *et al.*, 1977; Kaplan, 1981), there is a close relationship between the coagulation cascade and kinin formation. This fact, and the reports showing that some of the cells which can be activated by thrombin to produce prostaglandins also have specific receptors for bradykinin, stimulated us to examine the effect of bradykinin on bone resorption in an organ-culture system.

Abbreviations used: OAF, osteoclast activating factor; PGE_2, prostaglandin E_2; LDH, lactate dehydrogenase.

Experimental

Bradykinin (98% pure according to the manufacturer; prepared by solid-phase peptide synthe-

sis), fatty-acid-free serum albumin and phenolphthalein glucuronidate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Indomethacin was kindly provided by Merck, Sharp and Dohme, Haarlem, The Netherlands. CMRL 1066 medium was from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. $^{45}CaCl_2$ (42 Ci/g) was obtained from New England Nuclear Chemicals G.m.b.H., Dreieich, Germany.

The amount of bone resorption was measured by analysing the release of minerals from cultured mouse calvarial bones. The calvaria were dissected out aseptically from 6–7-day-old mice and divided along the sagittal suture into two halves. The calvarial halves were then transferred to dishes containing medium to which indomethacin had been added (1 μM final concn.). After 18–24 h the bones were washed three times in Tyrode's solution and then further washed by culturing in basic medium for 3 h. We have recently shown that this technique improves the subsequent response to parathyrin (parathyroid hormone) and thrombin by at least 100% as compared with the response in bones exposed to the stimulators without a preculture period in indomethacin (U. Lerner, unpublished work). The calvarial halves were then cultured separately on grids in stationary culture dishes essentially as described by Reynolds (1976). In one type of experiment, mineral release was monitored by quantifying the release of ^{45}Ca from bones prelabelled *in vivo* by injecting each mouse with 1.5 μCi of ^{45}Ca before killing the animal. After culture the amount of ^{45}Ca was analysed in the culture medium and in bones dissolved in HCl, and the mobilization of ^{45}Ca was expressed as the percentage of initial calculated activity. In these experiments the calvaria were cultured in 5.5 ml of medium. In a second type of experiment, calvarial halves were cultured in 2 ml of medium and the release of minerals was determined by analysing the concentration of Ca and P_i in medium before and after culture. Ca was analysed by atomic-absorption spectrophotometry (Willis, 1970) and P_i as described by Chen *et al.* (1956). In these experiments we also measured the activities of lysosomal and non-lysosomal enzymes in the bones and the culture media. The enzyme activites of the calvaria were liberated by incubating the bones in 0.1% (v/v) Triton X-100 for 24 h at 4°C. β-D-Glucuronidase (EC 3.2.1.31) was assayed with phenolphthalein glucuronidate as substrate (Vaes & Jacques, 1965). The activity of lactate dehydrogenase (LDH; EC 1.1.1.27) was determined by monitoring the oxidation rate of NADH at 340 nm and 25°C (Wróblewski & LaDue, 1955). The enzyme assays were performed under such conditions that the activity was directly proportional to both amount of enzyme and reaction time. The test compounds did not interfere with the assays. One unit refers to the decomposition of 1 μmol of substrate/min.

Results

Addition of bradykinin, at a concentration of 1 μM, to culture medium in which mouse calvaria were cultured for 72 h, resulted in a 2-fold increase in the release of ^{45}Ca (Table 1). The effect of bradykinin was dose-dependent, 0.03 μM producing a 1.4-fold increase of mineral mobilization. The stimulatory effect on ^{45}Ca release by 1 μM-bradykinin could be completely abolished by indomethacin (Table 1). In separate experiments we found that bradykinin (1 μM) not only stimulated the release of ^{45}Ca but also the mobilization of ^{40}Ca and P_i (Table 2). These findings suggest that the stimulatory effect of bradykinin on ^{45}Ca release was due to a cell-mediated process rather than to alteration in the passive exchange of isotope between bone and medium. From Table 2 it also appears that bradykinin caused an increased release of the lysosomal enzyme β-glucuronidase. In contrast, no effect of bradykinin on the release of the non-lysosomal enzyme LDH was registered (Table 2). The stimulation of the release of Ca, P_i and β-glucuronidase by bradykinin was completely abolished by addition of indomethacin to culture medium. Indomethacin, in conformity with previous reports (Lerner, 1982), also decreased the spontaneous release of Ca, P_i and β-glucuronidase (Table 2). However, no significant difference in the release of minerals and β-glucuronidase was found between the groups of bones treated with indomethacin in the presence and the absence of bradykinin. There was a statistically significant correlation between bone resorption, as assessed

Table 1. *Effect of bradykinin, in the absence and the presence of indomethacin, on the release of ^{45}Ca from cultured mouse calvarial bones*

Radioactivity labelled bones were dissected out, precultured for 24 h as described in the Experimental section and subsequently incubated with and without test substances for 72 h. After culture the radioactivity in the bones and the media was analysed and the percentage release of ^{45}Ca was calculated. Results are expressed as means ± S.E.M. for five experiments. [a,b]Significantly different from controls ([a]$P < 0.01$; [b]$P < 0.001$); [c]significantly different from bradykinin alone ($P < 0.001$).

Addition	Release of ^{45}Ca (%)
Control	17.5 ± 1.5
Bradykinin (0.03 μM)	25.7 ± 2.2[a]
Bradykinin (1 μM)	36.5 ± 2.0[b]
+ Indomethacin (1 μM)	17.9 ± 0.3[c]

Table 2. *Effect of bradykinin in the absence and the presence of indomethacin on the release of Ca, P_i, β-glucuronidase and LDH from mouse calvarial bones*

Non-labelled calvarial bones were dissected out, precultured for 24h as described in the Experimental section and subsequently incubated with and without test substances for 72h. After culture the concentrations of Ca and P_i in media were determined. In addition the activities of β-glucuronidase and LDH were assayed in extracts of bone and culture media. Total activities (medium + bone) of β-glucuronidase ($\times 10^{-5}$) were 25.2 ± 1.4 and 26.7 ± 2.1 units/half calvarium in the control and bradykinin groups respectively. Total activities of LDH were 226 ± 23 and 294 ± 19 units/half calvarium in the control and bradykinin groups respectively. Values are means ± S.E.M. for seven unpaired calvarial halves. [a,b]Significantly different from untreated controls ([a]$P<0.05$; [b]$P<0.01$); [c]significantly different from bradykinin alone ($P<0.01$).

Additions	Concn. (μM)	Ca (μg/half calvarium)	P_i (μg/half calvarium)	β-Glucuronidase (% of total)	LDH (% of total)
None (control)	–	13.82 ± 1.8	3.44 ± 0.7	12.9 ± 1.0	11.2 ± 1.0
Bradykinin	1	20.96 ± 2.3^a	8.14 ± 1.1^b	17.6 ± 0.8^a	11.3 ± 1.0
+ Indomethacin	1	11.19 ± 2.6^c	0.93 ± 0.5^c	8.2 ± 0.6^b	–
Indomethacin	1	–	-0.21 ± 1.0^b	9.3 ± 0.4^a	–

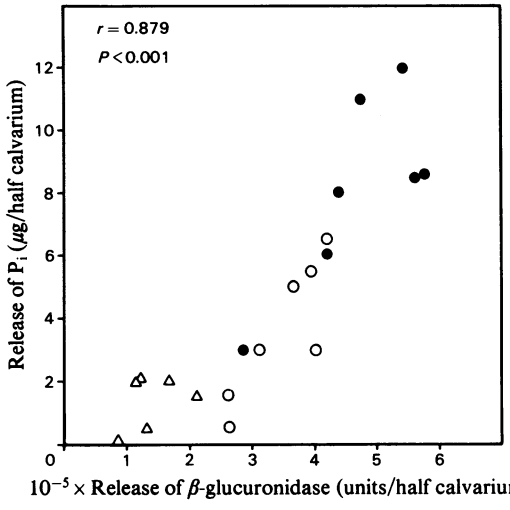

Fig. 1. *Correlation between the excretion of β-glucuronidase and the extent of bone resorption, as determined by the amount of P_i released from the calvarial bones*

The explants were cultured for 72h in the absence (○) and in the presence (●) of 1 μM-bradykinin. In addition one group of explants were cultured in the presence of 1 μM-bradykinin and 1 μM-indomethacin (△). After culture the concentration of P_i and the activity of β-glucuronidase was analysed in culture media and the release was calculated.

by release of P_i, and lysosomal-enzyme release (Fig. 1).

Discussion

The present results show that bradykinin can stimulate mineral mobilization and lysosomal-enzyme release in cultured mouse calvarial bones. That the mineral-mobilizing effect was due to active bone resorption was suggested by the findings indicating that bradykinin had no effect on the release of ^{45}Ca from devitalized bones (heated at 70°C for 5min; results not shown) and by our observation that, in bones treated with bradykinin, large holes were clearly visible after 72h of culture. Our finding that the release of lysosomal enzymes was stimulated in bones exposed to bradykinin further supports the view that bradykinin has a cell-mediated stimulatory effect in bone. Since bradykinin had no effect on the release of LDH, a cytosolic enzyme marker, the peptide seems to have a selective stimulatory effect on the release of lysosomal enzymes. The release of β-glucuronidase was significantly correlated with the amount of bone resorption, confirming previous biochemical and morphological observations indicating that the release of lysosomal enzymes is intimately associated with bone resorption (Eilon & Raisz, 1978; Lerner, 1980*a*,*b*; Vaes, 1980).

Bradykinin, most well known for its vasodilatory activity, can stimulate the production of prostaglandins in endothelial cells and fibroblasts by a receptor-mediated mechanism (Hong & Levine, 1976; Hong *et al.*, 1976; Hong, 1980; Becherer *et al.*, 1982; Whorton *et al.*, 1982). Since several products of arachidonic acid metabolism formed by the cyclo-oxygenase pathway can stimulate bone resorption in tissue culture (for a review, see Raisz & Martin, 1984), we tested the possibility that the mechanism of action by which bradykinin stimulates bone resorption is due to an increased endogenous production of prostaglandins. Indirect evidence for such a mechanism was provided by our finding that the cyclo-oxygenase inhibitor indomethacin completely blocked the effect of bradykinin on mineral mobilization and lysosomal-enzyme release. Although our results suggest that bone cells are equipped with receptors for bradykinin (the present paper) and thrombin (Gustafson & Lerner, 1983), we do not know at present in which cell(s) these receptors are located.

Such studies must await characterization of bradykinin and thrombin responses in isolated bone cells and fibroblasts. To characterize subtypes of bradykinin receptors involved, we need to investigate the bone-resorption-stimulating activity of other bradykinins, e.g. Lys-bradykinin produced by kallikrein-like proteinases from leucocytes (Movat *et al.*, 1973; Newball *et al.*, 1979) and Met-Lys-bradykinin formed under the influence of gland kallikreins (Habermann & Blennemann, 1964). Since bradykinin is formed in inflammatory processes, it is possible that this small peptide may be involved in the mechanisms responsible for activation of bone resorption associated with clinical inflammatory disorders. In areas of injury and inflammation, the blood-coagulation system may be activated via both the intrinsic and the extrinsic system, as elaborated in the introduction. As we have found that not only bradykinin, but also thrombin (Gustafson & Lerner, 1983), stimulated bone resorption, early as well as late factors generated during the coagulation cascade may influence bone tissue to develop a rarifying process.

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